High levels of mitochondrial DNA sequence divergence in isolated populations of freshwater snails of the genus *Goniobasis* Lea, 1862*

Robert T. Dillon, Jr. and Robert C. Frankis, Jr.

Department of Biology, College of Charleston, Charleston, South Carolina 29424, U.S.A., dillonr@cofc.edu

Abstract: In addition to their utility for phylogenetic reconstruction, mitochondrial sequence data have increasingly been applied to studies of species-level systematics. We amplified and sequenced a 709 bp fragment of the mitochondrial gene encoding cytochrome oxidase 1 and an approximately 530 bp fragment of the ribosomal large subunit (16S) gene for three individuals from each of three populations representing geographic races of the well-studied freshwater "prosobranch" snail *Goniobasis proxima*. By comparing intraspecific divergence to divergence in these same genes among *G. proxima* and the related *Goniobasis semicarinata* and *Goniobasis catenaria*, our purpose was to calibrate mitochondrial sequence data for application in future systematic studies of isolated, poorly-mobile molluscan populations in which genetic relationships may be less well understood. We identified four distinct haplotypes in the nine mitochondrial genomes of *G. proxima* amplified for each gene fragment, with a maximum likelihood sequence difference of 8.6%-16.9% for CO1 and 5.7%-18.7% for 16S. These levels of intraspecific divergence overlapped extensively with interspecific maximum likelihood differences, which ranged from 11.4%-17.7% for CO1 and 9.5%-16.5% for 16S. The extreme fragmentation that typically characterizes the population structure of freshwater gastropods, together with the ability of such populations to reach large size and great age, must be taken into consideration before systematic inference can be made on the basis of sequence divergence for these genes.

Keywords: 16S, CO1, *Elimia*, Virginia, Carolina

All major invertebrate taxa include poorly known groups in which the relationships between species are not clear. In freshwater and terrestrial molluscs, for example, species ranges may be fragmented into isolated populations and variation in shell morphology and other traditional characters may be negligible or subject to phenotypic plasticity. Thus as the tools of molecular genetics have become more accessible, malacologists have turned to DNA sequence data as a source of evidence by which biological species may be distinguished.

Before any new measurement tool can be employed efficiently, however, it must be calibrated. Levels of DNA sequence divergence should first be examined within and among populations for which specific relationships have previously been established by breeding studies or similarly direct means. If molecular data are to achieve ideal utility as criteria for species recognition, the maximum levels of sequence divergence among populations known to be conspecific should be less than the minimum sequence divergence between known, closely related species.

Among the most commonly sequenced genes in studies of molluscan population divergence are the mitochondrial genes encoding cytochrome oxidase I (CO1) and the large ribosomal subunit (16S). Sequence variation for the CO1 gene distinguishes unambiguously among species of the marine vesicomyid clams (Baco *et al.* 1999), the freshwater bivalve genera *Lasmigona* Rafinesque, 1831 (King *et al.* 1999) and *Corbicula* Megerle von Mühlfeld, 1811 (Renard *et al.* 2000), and the marine gastropod genera *Crepidula* Lamarck, 1799 (Collin 2000), *Notoacmaea* (Simison and Lindberg 1999), and *Hydrobia* Hartmann, 1821 (Wilke and Davis 2000, Wilke *et al.* 2000). Sequence variation for the 16S gene effectively discriminates among species in the marine bivalve genera *Ostrea* Linnaeus, 1758 (Ó Foighil *et al.* 1995, 1998, Jozefowicz and Ó Foighil 1998), and *Mercenaria* Schumacher, 1817 (Ó Foighil *et al.* 1996), and in the freshwater bivalve genera *Amblema* Rafinesque, 1820 (Mulvey *et al.* 1997) and *Dreissena* van Beneden, 1835 (Stepien *et al.* 1999). The 16S gene has also proven useful to distinguish species of land snails in the genera *Candidula* Kobelt, 1871 (Pfenninger and Magnin 2001), *Discus* Fitzinger, 1833 (Ross 1999) and *Cepaea* Held, 1837 (Thomaz *et al.* 1996).

In some situations, however, the level of sequence divergence within molluscan species has been found to exceed divergence among species. In the special case of doubly-uniparental inheritance, the male and female mitochondrial genomes within populations of *Mytilus* spp. and *Anodonta* spp. have diverged more than between same-sex compari-

* From the symposium "The Biology and Conservation of Freshwater Gastropods" presented at the annual meeting of the American Malacological Society, held 3-7 August 2002 in Charleston, South Carolina, USA.

sons of valid species (Rawson and Hilbish 1995, Hoeh *et al.* 1996). Sequence divergence among conspecific populations of the land snail *Mandarina* sp. from remote Pacific islands has proceeded to the extent that between-species variance seems to have been swamped (Chiba 1999).

We are aware of four prior works comparing the levels of DNA sequence divergence within and among conspecific populations of freshwater gastropods to divergence between related species. In three of these cases, researchers have reported an overlap between the maximum levels of divergence within species and the minimum divergence between species. In the pleurocerid fauna of Alabama, Lydeard *et al.* (1998) reported that 16S divergence ranged from 0% to 3.93% within species of *Goniobasis* Lea, 1862 (or *Elimia* H. and A. Adams, 1854) and from 0.3% to 11.08% between species. Populations representing different subspecies of the oriental pomatiopsid *Oncomelania hupensis* (Gredler, 1881) may show CO1 sequence differences exceeding those reported between the related pomatiopsid genera *Gammatricula* Davis and Liu, 1990 and *Tricula* Benson, 1843 (Davis *et al.* 1998). Hershler *et al.* (1999b) reported that the CO1 divergence between two Death Valley populations of the hydrobiid *Tryonia variegata* Hershler and Sada, 1987 was equal to or greater than the level observed in most other comparisons among eight species of *Tryonia* Stimpson, 1865. The subsequent results of Hershler *et al.* (1999a) on a larger sample of hydrobiids from the American southwest seemed to cast doubt on previous assumptions regarding specific relationships in this group. In any case, it is possible that the evolution of DNA sequences in freshwater gastropods may be more similar to that described by Chiba (1999) for island populations of the land snail *Mandarina* sp. than to the bivalves, marine gastropods, or even most terrestrial gastropods that have attracted the bulk of previous study.

At the level of its population genetics, the pleurocerid *Goniobasis proxima* (Say, 1825) is among the best known of all freshwater gastropods. The purpose of this paper was to assess sequence variation for the mitochondrial CO1 and 16S genes within and among populations of *G. proxima* and compare the intraspecific values obtained to interspecific values from two other well-characterized *Goniobasis* species known to be related. Our purpose is to confirm the small body of previously published evidence suggesting that populations of freshwater snails may be so old, large, and isolated that intrapopulation sequence divergence is liable to swamp interpopulation sequence divergence in two of the mitochondrial genes most commonly examined by malacologists using the tools of molecular genetics.

The Pleuroceridae is a holarctic family of freshwater "prosobranch" gastropods that has diversified extensively in the rivers and streams of the American southeast. Populations are perennial and may reach great densities, locally hundreds per square meter. Reproduction is entirely sexual, as far as is known. The biology of the Pleuroceridae has been reviewed by Dillon (2000). *Goniobasis proxima* is a common pleurocerid inhabitant of small softwater streams in the piedmont and mountains from southern Virginia to northern Georgia, on both sides of the eastern continental divide. Populations are isolated both by intervening mountain ranges and by larger rivers, to which the snail does not seem adapted. A sample of 25 populations from a 20,000 km^2 area straddling the borders of Virginia, North Carolina, and Tennessee showed extreme divergence in both morphology and allozyme frequencies; some pairs of populations sharing no alleles at multiple enzyme loci (Dillon 1984a). Three races of *G. proxima* have been recognized (A, B, and C) on the basis of shell morphology and allozyme divergence inhabiting different parts of the range (Dillon and Davis 1980, Dillon 1984b). Transplant experiments (Dillon 1988a) and artificial introductions (Dillon 1986) have, however, uncovered no evidence of reproductive isolation among any of these populations.

As genetically diverse as *Goniobasis proxima* may be, its levels of interpopulation divergence do not approach those recorded among other well-characterized species, such as *Goniobasis semicarinata* (Say, 1829) (Dillon and Davis 1980) or *Goniobasis catenaria* (Say, 1822) (Dillon and Reed 2002). *Goniobasis semicarinata* is primarily an inhabitant of the American interior, ranging through Ohio, Indiana, and Kentucky. Its biology is similar to that of *G. proxima*, although it bears a heavier shell and is restricted to harder water and lower elevations. The southern limit of *G. semicarinata* contacts the northern border of the range of *G. proxima* in the New River drainage of Virginia. *Goniobasis catenaria* inhabits streams and rivers on the southern and eastern borders of the *G. proxima* range (Dillon and Keferl 2000). There are several subspecies, one of which (*G. catenaria dislocata*) bears a shell distinguishable from that of *G. proxima* only by faint axial costae. The 2n = 36 karyotype of *G. catenaria* is not strikingly different from the 2n = 34 karyotype of *G. proxima* (Dillon 1989, 1991), but there is no evidence of hybridization between the two species, even in close contact (Dillon and Reed 2002). So analyzed together, the *Goniobasis* fauna of the southeastern United States would seem an excellent model upon which to gauge the utility of mitochondrial sequence data for species discrimination in freshwater gastropods, and perhaps in poorly-mobile freshwater invertebrates more generally.

METHODS

Study populations

Although separated by less than 120 km over land, our three populations of *Goniobasis proxima* shared no freshwa-

ter connection (Fig. 1). Our Race A sample came from a tributary of the Yadkin River, which drains south to the Atlantic through the Pee Dee system, our sample of Race B was from a tributary of the New River, flowing west to the Mississippi through the Ohio River system, and our sample of Race C was from a small tributary of the Dan River, flowing east to the Atlantic through the Roanoke River system. Our sample of *Goniobasis semicarinata* was collected from a small tributary of the New River only 50 km east of our *G. proxima* Race C. Our *Goniobasis catenaria* came from a tributary of the Santee River, which flows through South Carolina to the Atlantic approximately 350-400 km south of the other four populations.

Detailed locality data are as follows: *Goniobasis proxima* Race A-Naked Creek at NC 1154 bridge, 5.2 km N of Furguson, Wilkes Co., NC. *Goniobasis proxima* Race B-Cripple Creek at Va 671 bridge, 3.7 km E of Cedar Springs, Wythe Co., VA. *Goniobasis proxima* race C-Nicholas Creek at Va 623 bridge, 5.2 km SW of Ferrum, Franklin Co., VA. *Goniobasis semicarinata*-Little Pine Run at Va 100 bridge, 12 km S of Pulaski, Pulaski Co, VA. *Goniobasis catenaria dislocata*-the head of Chapel Branch, Santee, Orangeburg Co., SC. Allozyme data and maps locating these populations have been published as follows: Race A is "Yad" of Dillon and Davis (1980) and Dillon and Reed (2002) or "Yad1" of Dillon (1984a, 1988b). Race B is "Crip" of Dillon and Davis (1980) and Dillon (1984a, 1988a). Race C is "Phlp" of Dillon (1984a). Our *Goniobasis semicarinata* is population "Pine" of Dillon and Davis (1980) and our *G. catenaria dislocata* is population "Sant" of Dillon and Reed (2002).

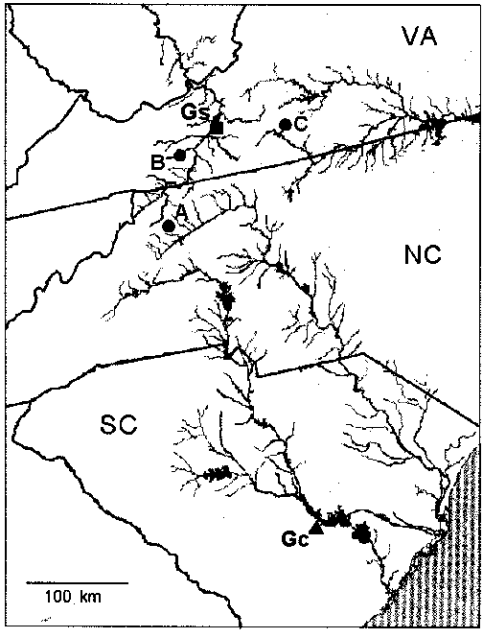

Figure 1. A portion of the southeastern United States, showing drainage relationships among sample sites. The circles are populations of *Goniobasis proxima* (A, B, C), the square is a population of *Goniobasis semicarinata* (Gs), and the triangle is a population of *Goniobasis catenaria* (Gc).

We analyzed three individuals from each race of *G. proxima* and one individual from each of the other two species. Thus a total of 11 fragments were amplified and sequenced for each of the two mitochondrial genes examined here.

Laboratory methods

Total cellular DNA was obtained using either fresh or previously frozen samples of whole buccal mass (~20 mg). Following proteinase K digestion of the tissue, the DNA was extracted using either a DNeasy Tissue Kit (Qiagen) or by two phenol and two chloroform extractions followed by ethanol precipitation. The optimum DNA concentration for PCR of each of the samples was determined empirically.

We amplified a 709 bp fragment of the mitochondrial cytochrome oxidase I gene using the "universal" CO1 primers of Folmer *et al.* (1994): 5′-ggtcaacaaatcataaagatattgg-3′ and 5′-taaacttcagggtgaccaaaaaatca-3′. Our 525-532 bp fragment from the 5′ half of the 16S mitochondrial rDNA gene was amplified using primers "SNL002" and "SNL448" of Lydeard *et al.* (1997, 1998), the former trimmed slightly to afford a better match of annealing temperatures: 5′-aaatgattatgctaccttt-3′ and 5′-gaaatttcattcgcactag-3′. The primer "16sar-L" (or L2510) commonly used as a starting point to amplify the 3′ half of the mitochondrial 16S gene (Palumbi *et al.* 1991) is encountered around bases 410-430 of the sequences we determined in the present study. Lydeard *et al.* (1998) reported that the 5′ half of the pleurocerid 16S gene shows greater variability than the 3′ half more usually sequenced by other workers.

A typical amplification reaction of 50 μL contained 1.5 μL of DNA (the optimum amounts generally ranged between 50 and 200 ng, determined empirically), 100 mM Tris (pH 9), 50 mM KCl, 1.5 units of Taq DNA polymerase, 150 μM of each of the four deoxynucleotide triphosphates, and 0.2 μM of each primer. PCR amplification was by a 15 min 95°C activation step followed by 30 cycles, each consisting of 45 sec at 94°C, 1 min at 45°C, and 1 min at 72°C. Upon completion of the 30 cycles a 10 min 72°C incubation was performed to extend uncompleted strands. Amplification of

fragments of the expected size was verified by agarose gel electrophoresis. The PCR-amplified DNA was prepared for sequencing using a QIAquick PCR purification kit (Qiagen). Cycle sequencing was performed by the Medical University of South Carolina Biotechnology Resource Laboratory. The PCR product was sequenced twice for both strands.

Analysis

Our initial alignments of the four sequence fragments obtained for each individual were performed using the "Web align" feature of Biowire Jellyfish (version 1.5, Biowire.com) with default settings. Final alignments (between individuals) were performed online with program *blastn* through the "Blast two sequences" utility available from the National Center for Biotechnology Information (Tatusova and Madden 1999, NCBI 2003). The apparently high frequency of indels in our 16S data prompted us to lower the gap opening penalty from 10 to 2 and the gap extension penalty from 2 to 1. The strong A/T bias also observable in our 16S data prompted us to de-select the low complexity filter.

We translated our CO1 sequence fragments with the invertebrate mitochondrial code and a +3 lag using Biowire Jellyfish, then aligned the resulting amino acid sequences pairwise with the *blastp* program, also available online through the NCBI "Blast two sequences" utility (NCBI 2003). All *blastp* parameters were set to default, except that the low complexity filter was de-selected.

We recorded the simple percent nucleotide difference ("p distance") between each unique pair of sequences as one minus the identity returned by the pairwise BLAST utility, extending through the entire (unvaried) primer region on each end. Where indels had apparently yielded two sequences of different lengths, the length of the larger sequence served as denominator in the calculation of identity. Thus the impact of each indel was weighted by its length.

We also calculated maximum likelihood (ML) distances among our sequence fragments using the DNADIST program available in PHYLIP (version 3.573c, Felsenstein 1995). Both the base composition frequencies and transition:transversion ratios were determined empirically. A single joint analysis was performed for the CO1 data. Because indels are scored as missing data (rather than as mismatches) in the calculation of ML distances, however, their accumulated effects across the diverse 16S sequences we ultimately obtained would have resulted in substantial loss of data upon multiple alignment. We therefore elected to calculate ML distances among 16S sequences pairwise in multiple separate runs, rather than in a single joint analysis.

For parsimony analysis, we performed a conventional multiple alignment of our 16S sequences using BioEdit version 5.0.9 (Hall 1999) and concatenated each 16S sequence (elongated by multiple insertions) to its corresponding CO1 sequence. We then analyzed the combined data set using PAUP* version 4.0b10 (Swofford 2002), setting *Goniobasis semicarinata* as root, *Goniobasis catenaria* as root, and *semicarinata* + *catenaria* as root, with 1,000 bootstrap replicates.

RESULTS

Results for the CO1 and the 16S genes were similar in many respects. All three individuals of *Goniobasis proxima* from population A yielded identical CO1 and 16S haplotypes, as did all three individuals from population B. Population C yielded two strikingly different CO1 haplotypes and two strikingly different 16S haplotypes. For both genes, the haplotype carried by two individuals was designated "C1" and the haplotype carried by the third snail was designated "C2." The two other species, *Goniobasis semicarinata* and *Goniobasis catenaria*, also yielded distinct haplotypes, resulting in six unique sequence fragments for each gene. The total of 12 unique sequences has been entered in GenBank, accession numbers AY063464-AY063475.

The total length, from the 5′ beginning of the first primer to the 3′ end of the second primer, was 709 bp for all six unique CO1 sequence fragments. Table 1 shows that the simple uncorrected nucleotide difference between the four sequences of *Goniobasis proxima* ranged from 8.0% between populations A and B to 14.7% between the two sequences identified in population C. This translated to an amino acid difference of 1.3%-5.5%. Interspecific divergence was not strikingly different from intraspecific divergence, evaluated at the maximum. The uncorrected difference between *Goniobasis semicarinata* and the other species, and between *Goniobasis catenaria* and the other species, ranged from 10.2%-15.2% as nucleotides or 0.4%-5.1% as amino acids.

Combined over the six unique CO1 sequences we obtained, the base frequencies were 24%A, 19%C, 20%G, and

Table 1. Comparisons of six mitochondrial CO1 sequence fragments amplified from three species of *Goniobasis*. Above the diagonal are the percent differences (p distances) of nucleotide bases (709 in the denominator, including both primers) and below the diagonal are percent amino acid differences (235 in the denominator).

	A	B	C1	C2	*G.s.*	*G.c.*
G. proxima A		8.0	14.0	14.1	11.7	10.9
G. proxima B	4.3		12.7	14.5	10.2	10.3
G. proxima C1	2.1	5.5		14.7	13.5	15.2
G. proxima C2	1.7	5.1	1.3		13.5	14.4
G. semicarinata	0.4	3.8	1.7	1.3		12.1
G. catenaria	0.9	5.1	3.0	2.1	1.3	

37%T. Pairwise transition : transversion (Ts : Tv) ratios are shown in Table 2, the overall empirical ratio being 4.14. Corrected by the base frequencies and the Ts : Tv ratio, the six sequences are arranged by their maximum likelihood distances in Fig. 2.

The distance from the 5′ end of the leading primer to the 3′ end of the trailing primer for the 16S fragment amplified in this study ranged from 524–532 bp for the six unique sequences we obtained. Table 3 shows that uncorrected sequence differences ranged from 6.1%–17.1% among the populations of *Goniobasis proxima*, with 1-4 indels apparent for each comparison. Evaluated at the maximum, this is again not strikingly different from the levels of divergence between species, which ranged from 9.3%–17.9%.

Variable bases did not seem equally distributed across the approximately 530 bases of the 16S fragment we amplified, but rather seemed localized, as one might expect from the stem-and-loop structures assumed by ribosomal subunits. Apparent Ts : Tv ratios were generally low, approaching unity in several instances (Table 2). Across all six unique 16S sequence fragments (3,170 bases) the base frequencies were 35% A, 36% T, 13% C, and 16% G. Corrected by base composition and pairwise Ts : Tv ratios, the sequences are diagramed by their maximum likelihood distances in Fig. 3.

Multiple alignment elongated the joint 16S product to 545 bases by multiple insertion, which when concatenated with the 709 CO1 bases yielded a combined sequence of 1,254 characters. Of these 928 were constant, 207 were parsimony-uninformative, and 119 were parsimony-informative. Phylogenetic analysis with both *Goniobasis semicarinata* and *Goniobasis catenaria* specified as roots returned single trees of length 478 (CI = 0.8159, HI = 0.1841), both depicting *Goniobasis proxima* as paraphyletic. The third analysis, combining *G. semicarinata* and *G. catenaria*, could not be rooted such that the outgroup was monophyletic, and collapsed to yield the single tree rooted by *G. semicarinata* alone (Fig. 4).

Table 2. Apparent transition : transversion ratios in comparisons of six mitochondrial sequence fragments amplified from 3 species of the genus *Goniobasis*. Data for the CO1 gene are above the diagonal, and those for the 16S gene are given below.

	A	B	C1	C2	*G.s.*	*G.c.*
G. proxima A		16.7	3.50	3.50	4.57	3.81
G. proxima B	6.00		3.05	3.86	3.80	3.50
G. proxima C1	3.86	8.00		2.52	2.25	3.12
G. proxima C2	2.35	2.33	2.15		2.56	2.33
G. semicarinata	3.42	2.83	2.38	1.31		3.05
G. catenaria	4.18	3.46	2.86	1.18	1.29	

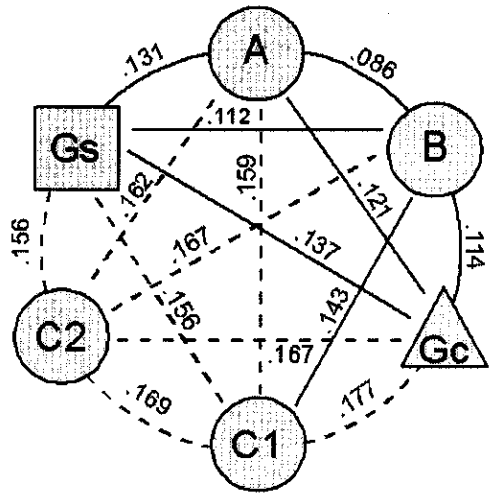

Figure 2. The six unique mitochondrial CO1 sequence fragments diagramed by their maximum likelihood distances. The circles are *Goniobasis proxima* (A, B, C1, C2), the square is *Goniobasis semicarinata* (Gs), and the triangle is *Goniobasis catenaria* (Gc). Thick segments join haplotypes that are less than 10% different, thin segments connect haplotypes ranging from 10%–15% different, and dashed segments join haplotypes of greater than 15% maximum-likelihood distance.

DISCUSSION

The level of sequence divergence we observed within and among conspecific populations of *Goniobasis proxima* was exceptionally high. A review of the molluscan literature suggests that intraspecific divergence in either of the genes we examined here typically ranges no higher than 5%. This generalization holds true for marine gastropods (Simison and Lindberg 1999, Collin 2000, Hamm and Burton 2000, Wilding *et al.* 2000, Wilke and Davis 2000), marine bivalves (Geller *et al.* 1993, Ó Foighil *et al.* 1996, Chase *et al.* 1998, Ó Foighil *et al.* 1998, Baco *et al.* 1999), and freshwater bivalves (King *et al.* 1999, Stepien *et al.* 1999, Renard *et al.* 2000). A notable exception occurs in the sex-specific haplotypes of certain bivalves, which may differ by as much as 30% (Hoeh *et al.* 1996, 1997). Levels of sequence divergence among conspecific populations of land snail are also generally reported to reach maxima higher than the 5% typical for most molluscs: 5.3% in *Partulina* Pfeiffer, 1854 (Thacker and Hadfield 2000), 8% in *Candidula* (Pfenninger and Magnin 2001), 8.4% in *Discus* (Ross 1999), 9.5% in *Euhadra* Pilsbry, 1890

Table 3. Comparisons of six mitochondrial 16S sequence fragments amplified from three species of *Goniobasis*. The diagonal gives the sequence length, including both primer regions. Above the diagonal are percent nucleotide differences (p distances), where the denominator is the sum of the sequence length and its corresponding apparent number of indel bases. Below the diagonal is the number of indels, recorded as the apparent number of deletions in the column sequence (total bases) over the number of deletions in the row sequence (total bases).

	A	B	C1	C2	*G.s.*	*G.c.*
G. proxima A	528	6.1	7.4	17.1	11.2	12.5
G. proxima B	0/3(3)	525	6.2	16.4	9.3	12.9
G. proxima C1	1(2)/0	3(5)/0	530	14.5	9.8	12.8
G. proxima C2	3(5)/2(2)	4(6)/0	4(7)/2(6)	531	14.7	17.9
G. semicarinata	0/2(4)	1(1)/2(2)	0/3(6)	1(1)/4(8)	524	11.8
G. catenaria	2(7)/3(3)	3(8)/1(1)	4(8)/4(6)	8(12)/3(11)	3(8)/0	532

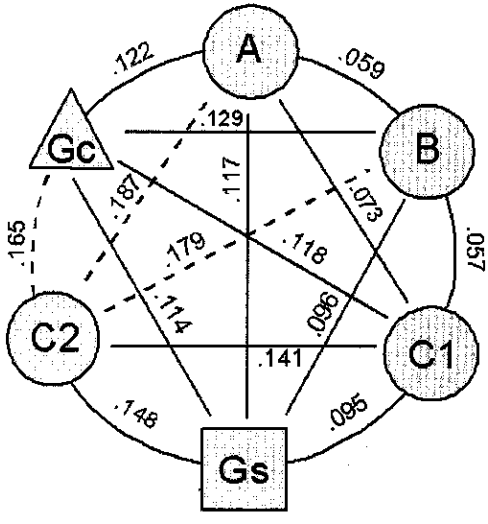

Figure 3. The six unique mitochondrial 16S sequence fragments diagramed by their maximum likelihood distances. The circles are *Goniobasis proxima* (A, B, C1, C2), the square is *Goniobasis semicarinata* (Gs), and the triangle is *Goniobasis catenaria* (Gc). Thick segments join haplotypes that are less than 10% different, thin segments connect haplotypes ranging from 10%-15% different, and dashed segments join haplotypes of greater than 15% maximum-likelihood distance.

(Hayashi and Chiba 2000), 11.1% in *Helix* Linné, 1758 (Guiller *et al.* 2001), 12.9% in *Cepaea* (Thomaz *et al.* 1996), 13% in *Arianta* Leach *in* Turton, 1831 (Haase *et al.* 2003) and 18.7% in *Mandarina* (Chiba 1999).

It is difficult to generalize regarding the levels of sequence divergence previously reported among conspecific populations of freshwater gastropods. Lydeard *et al.* (1998) compared 8 individual *Goniobasis* (or *Elimia*) *carinocostata* (Lea, 1845) from five sites and obtained a maximum 16S sequence divergence of 3.9%. The maximum CO1 divergence among conspecific individuals of *Tryonia* from Death Valley was 5.2% (Hershler *et al.* 1999b). Within subspecies of *Oncomelania hupensis*, the maximum CO1 divergence seemed to average around 2.1% (Davis *et al.* 1999), while maxima between the subspecies reached 14.2-15.3% (Davis *et al.* 1998).

In *Goniobasis proxima*, we have discovered maximum divergences of p = 14.7% or ML = 16.9% for the CO1 gene and p = 17.1% or ML = 18.7% for the 16S gene. These rank among the highest intraspecific value for sequence divergence yet reported for molluscs. Thomaz *et al.* (1996) suggested four (overlapping) explanations for the high levels of sequence divergence they observed among populations of the land snail *Cepaea nemoralis* (Linné, 1758): a large effective population size, fragmentation of the range into isolated demes, disruptive selection, and a systemically higher rate of mitochondrial evolution. Of these four, the factor most conspicuously shared by the land and freshwater snails (but not by marine molluscs or by bivalves generally) is population fragmentation. Our three populations of *G. proxima* shared no connection through water and may have been isolated for millions of years.

Our sample of three Race C snails included a pair of strikingly divergent haplotypes for both the genes we examined. The population from which these snails were drawn ("Phlp" of Dillon 1984a) is homogeneous both in morphology and in gene frequency at seven enzyme-encoding nuclear genes. Apparently, conspecific pleurocerids sampled from adjacent rocks may show more mitochondrial DNA sequence divergence than verifiably distinct species isolated by 400 km overland. Data addressing the possible existence of intermediate forms between these two diverse mitochondrial haplotypes in the Phlp population would cast light on whether such great genetic diversity may have evolved *in situ* or might reflect the admixture of two previously isolated populations.

The levels of sequence divergence we observed within and among populations of *Goniobasis proxima* exceeded their (interspecific) divergence with *Goniobasis semicarinata* and *Goniobasis catenaria* in many cases. For the CO1 gene, haplotypes A and B were more similar to *G. semicarinata* or

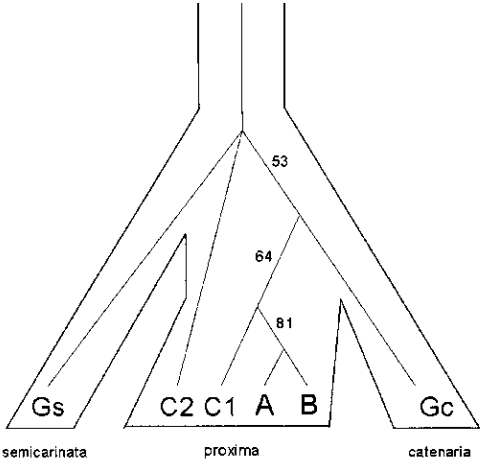

Figure 4. The single most parsimonious tree (CI = 0.816, HI = 0.184) returned by phylogenetic analysis where *Goniobasis semicarinata* was specified as root. Bootstrap values (percent of 1,000 replicates) are indicated at the nodes.

G. catenaria than to either C1 or C2, regardless of the metric employed (Table 1, Fig. 2). For the 16S gene, haplotype C2 was so strikingly distinct as to render all the other five haplotypes neighbors by comparison (Table 3, Fig. 3).

Phylogenetic analysis of the combined data set under the parsimony criterion suggested that *Goniobasis proxima* is paraphyletic with respect to either of its congeners in the American southeast. This may be a consequence of "lineage sorting" as depicted in Fig. 4 (Takahata and Nei 1985, Rosenberg 2003). The bootstrap support for most of the branches in the phylogeny was not high, however, and the tree topology may reflect more noise than signal.

There is some evidence that the sites available for variation in these genes may be approaching saturation in this sample of populations of *Goniobasis*. Haplotypes A and B appeared to be the most similar by almost all measures, and displayed an exceptionally high Ts : Tv ratio (Table 2). Omitting the A/B comparison for CO1, the overall average Ts : Tv ratio across the six populations droped from 4.14 to 3.24. The relationship between B and C1 was also apparently close (judging from 16S data) and characterized by a high Ts : Tv ratio. Setting aside these two individual comparisons, however, the Ts : Tv ratios we obtained were generally less than 4 : 1. It is interesting to note that the greatest difference in CO1 amino acid sequence (5.5%) was posted between *Goniobasis proxima* B and C1, which the maximum likelihood analysis of 16S sequence data suggested as the most similar pair of populations. Our observation that interspecific differences in amino acid sequence were strikingly lower than intraspecific differences in most cases (Table 1) further suggests that sequence divergence may be approaching saturation in these highly isolated populations of freshwater snails.

Under such circumstances, systematic inference must be made with care. The existence of a high level of sequence divergence can apparently be interpreted as little evidence that a pair of freshwater snail populations is specifically distinct. The only other pleurocerid populations for which data are available on both gene frequencies at nuclear loci and mitochondrial sequence divergence are the *Leptoxis* spp. of Alabama. Lydeard *et al.* (1997) reported up to 19.4% sequence divergence for the 16S gene among single individuals of three nominal species: *Leptoxis ampla* (Anthony, 1855), *Leptoxis taeniata* (Conrad, 1834), and *Leptoxis picta* (Conrad, 1834). These results appeared incompatible with a much larger data set on gene frequencies at nine enzyme-encoding loci in six populations (30 individuals per population), which suggested that the three nominal species might be conspecific (Dillon and Lydeard 1998). It now seems apparent that an uncorrected sequence difference as high as 19% for the 16S gene does not necessarily contradict the conspecific hypothesis for pleurocerid populations.

An observation of low levels of divergence may constitute some evidence that a pair of populations is conspecific, however. In addition to their data on the three individuals of *Leptoxis* spp. noted above, Lydeard and his colleagues (Lydeard *et al.* 1997, 1998, Holznagel and Lydeard 2000) have reported sequence data from the 16S rDNA gene for over 30 species (representing five genera) of North American pleurocerid snails. Their sampling effort has focused on the Mobile Basin of Alabama because of its putatively high pleurocerid diversity, and has been directed toward elucidating higher-level evolutionary relationships. Like most of the American freshwater gastropod fauna, however, the taxonomy of the Alabama Pleuroceridae predates the modern synthesis, being based almost entirely upon minor attributes of the shell. The maximum divergence among the individual snails representing seven nominal species of Alabama *Goniobasis* sequenced by Lydeard *et al.* (1997) was only 7.89% (uncorrected), with many pairwise values less than 2%. A critical re-examination of the biological species of pleurocerid snails in the Mobile Basin, and throughout most of North America, is long overdue.

ACKNOWLEDGMENTS

Laboratory assistance was provided by Shela Patel, Jennifer Ivey, Zachery Evans, and Jessica Pease. We thank Allan

Strand, Chuck Lydeard, and especially Amy Wethington for technical advice and assistance. Allan Strand and Matthias Glaubrecht provided helpful comments on an earlier draft of the manuscript. This research was funded by grants from the Department of Biology and College of Charleston R&D Committees.

LITERATURE CITED

Baco, A. R., C. R. Smith, A. S. Peek, G. K. Roderick, and R. C. Vrijenhoek. 1999. The phylogenetic relationships of whale-fall vesicomyid clams based on mitochondrial CO1 DNA sequences. *Marine Ecology Progress Series* **182**: 137-147.

Chase, M. R., R. J. Etter, M. A. Rex, and J. M. Quattro. 1998. Bathymetric patterns of genetic variation in a deep-sea protobranch bivalve, *Deminucula atacellana*. *Marine Biology* **131**: 301-308.

Chiba, S. 1999. Accelerated evolution of land snails *Mandarina* in the oceanic Bonin Islands: Evidence from mitochondrial DNA sequences. *Evolution* **53**: 460-471.

Collin, R. 2000. Phylogeny of the *Crepidula plana* (Gastropoda: Calyptraeidae) cryptic species complex in North America. *Canadian Journal of Zoology* **78**: 1500-1514.

Davis, G. M., T. Wilke, C. Spolsky, C-P. Qiu, D-C. Qiu, M-Y. Xia, Y. Zhang, and G. Rosenberg. 1998. Cytochrome oxidase I-based phylogenetic relationships among the Pomatiopsidae, Hydrobiidae, Rissoidae, and Truncatellidae. *Malacologia* **40**: 251-266.

Davis, G. M., T. Wilke, Y. Zhang, X-J. Xu, C-P. Qiu, C. Spolsky, D-C. Qiu, Y. Li, M-Y. Xia, and Z. Feng. 1999. Snail-*Schistosoma*, *Paragonimus* interactions in China: Population ecology, genetic diversity, coevolution and emerging diseases. *Malacologia* **41**: 355-377.

Dillon, R. T., Jr. 1984a. Geographic distance, environmental difference, and divergence between isolated populations. *Systematic Zoology* **33**: 69-82.

Dillon, R. T., Jr. 1984b. What shall I measure on my snails? Allozyme data and multivariate analysis used to reduce the nongenetic component of morphological variance in *Goniobasis proxima*. *Malacologia* **25**: 503-511.

Dillon, R. T., Jr. 1986. Inheritance of isozyme phenotype at three loci in the freshwater snail, *Goniobasis proxima*: Mother-offspring analysis and an artificial introduction. *Biochemical Genetics* **24**: 281-290.

Dillon, R. T., Jr. 1988a. Evolution from transplants between genetically distinct populations of freshwater snails. *Genetica* **76**: 111-119.

Dillon, R. T., Jr. 1988b. Minor human disturbance influences biochemical variation in a populaton of freshwater snails. *Biological Conservation* **43**: 137-144.

Dillon, R. T., Jr. 1989. Karyotypic evolution in pleurocerid snails: I. Genomic DNA estimated by flow cytometry. *Malacologia* **31**: 197-203.

Dillon, R. T., Jr. 1991. Karyotypic evolution in pleurocerid snails: II. *Pleurocera*, *Goniobasis*, and *Juga*. *Malacologia* **33**: 339-344.

Dillon, R. T., Jr. 2000. *The Ecology of Freshwater Molluscs*. Cambridge University Press, Cambridge.

Dillon, R. T., Jr., and G. M. Davis. 1980. The *Goniobasis* of southern Virginia and northwestern North Carolina: Genetic and shell morphometric relationships. *Malacologia* **20**: 83-98.

Dillon, R. T., Jr., and E. P. Keferl. 2000. A survey of the pleurocerid gastropods of South Carolina. *In:* R. A. Tankersley, D. I. Warmolts, G. T. Watters, B. J. Armitage, P. D. Johnson, and R. S. Butler, eds., *Freshwater Mollusk Symposium Proceedings*, Ohio Biological Survey, Columbus. Pp. 153-160.

Dillon, R. T., Jr., and C. Lydeard. 1998. Divergence among Mobile Basin populations of the pleurocerid snail genus, *Leptoxis*, estimated by allozyme electrophoresis. *Malacologia* **39**: 113-121.

Dillon, R. T., Jr., and A. J. Reed. 2002. A survey of genetic variation at allozyme loci among *Goniobasis* populations inhabiting Atlantic drainages of the Carolinas. *Malacologia* **44**: 23-31.

Felsenstein, J. 1995. *PHYLIP (Phylogeny Inference Package) version 3.57c*. University of Washington, Seattle [Privately distributed].

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294-299.

Geller, J. B., J. T. Carlton, and D. A. Powers. 1993. Interspecific and intrapopulation variation in mitochondrial ribosomal DNA sequences of *Mytilus* spp. (Bivalvia: Mollusca). *Molecular Marine Biology and Biotechnology* **2**: 44-50.

Guiller, A., M. A. Coutellec-Vreto, L. Madec, and J. Deunff. 2001. Evolutionary history of the land snail *Helix aspersa* in the western Mediterranean: Preliminary results inferred from mitochondrial DNA sequences. *Molecular Ecology* **10**: 81-87.

Haase, M., B. Misof, T. Wirth, H. Baminger, and B. Baur. 2003. Mitochondrial differentiation in a polymorphic land snail: Evidence for Pleistocene survival within the boundaries of permafrost. *Journal of Evolutionary Biology* **16**: 415-428.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Hamm, D. E. and R. S. Burton. 2000. Population genetics of black abalone, *Haliotis cracherodii*, along the central California coast. *Journal of Experimental Marine Biology and Ecology* **254**: 235-247.

Hayashi, M. and S. Chiba. 2000. Intraspecific diversity of mitochondrial DNA in the land snail *Euhadra peliomphala* (Bradybaenidae). *Biological Journal of the Linnean Society* **70**: 391-401.

Hershler, R., H-P. Liu, and M. Mulvey. 1999a. Phylogenetic relationships within the aquatic snail genus *Tryonia*: Implications for biogeography of the North American southwest. *Molecular Phylogenetics and Evolution* **13**: 377-391.

Hershler, R., M. Mulvey, and H-P. Liu. 1999b. Biogeography in the Death Valley region: Evidence from springsnails (Hydrobiidae: *Tryonia*). *Zoological Journal of the Linnean Society* **126**: 335-354.

Hoeh, W. R., D. T. Stewart, C. Saavedra, B. W. Sutherland, and E.

Zouros. 1997. Phylogenetic evidence for role-reversals of gender-associated mitochondrial DNA in *Mytilus* (Bivalvia: Mytilidae). *Molecular Biology and Evolution* **14**: 959-967.

Hoeh, W. R., D. T. Stewart, B. W. Sutherland, and E. Zouros. 1996. Multiple origins of gender-associated mitochondrial DNA lineages in bivalves (Mollusca: Bivalvia). *Evolution* **50**: 2276-2286.

Holznagel, W. E., and C. Lydeard. 2000. A molecular phylogeny of North American Pleuroceridae (Gastropoda: Cerithioidea) based on mitochondrial 16s rDNA sequences. *Journal of Molluscan Studies* **66**: 233-257.

Jozefowicz, C. J., and D. Ó Foighil. 1998. Phylogenetic analysis of southern hemisphere flat oysters based on partial mitochondrial 16s rDNA gene sequences. *Molecular Phylogenetics and Evolution* **10**: 426-435.

King, T. L., M. S. Eackles, B. Gejetvaj, and W. R. Hoeh. 1999. Intraspecific phylogeography of *Lasmigona subviridis* (Bivalvia: Unionidae): Conservation implications of range discontinuity. *Molecular Ecology* **8**: 65-78.

Lydeard, C., W. E. Holznagel, J. Garner, P. Hartfield, and J. M. Pierson. 1997. A molecular phylogeny of Mobile River drainage basin pleurocerid snails (Caenogastropoda: Cerithioidea). *Molecular Phylogenetics and Evolution* **7**: 117-128.

Lydeard, C., J. H. Yoder, W. E. Holznagel, F. G. Thompson, and P. Hartfield. 1998. Phylogenetic utility of the 5'-half of mitochondrial 16S rDNA sequences for inferring relationships of *Elimia* (Cerithioidea: Pleuroceridae). *Malacologia* **39**: 183-193.

Mulvey, M., C. Lydeard, D. L. Pyer, K. M. Hicks, J. Brim-Box, J. D. Williams, and R. S. Butler. 1997. Conservation genetics of North American freshwater mussels *Amblema* and *Megalonaias*. *Conservation Biology* **11**: 868-878.

NCBI [National Center for Biotechnology Information, U.S. National Institutes of Health]. 2003. Blast two sequences utility. Available at: http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html 20 August 2003.

Ó Foighil, D., P. M. Gaffney, and T. J. Hilbish. 1995. Differences in mitochondrial 16S ribosomal gene sequences allow discrimination among American [*Crassostrea virginica* (Gmelin)] and Asian [*C. gigas* (Thunberg) *C. ariakensis* Wakiya] oyster species. *Journal of Experimental Marine Biology and Ecology* **192**: 211-220.

Ó Foighil, D., P. M. Gaffney, A. E. Wilbur, and T. J. Hilbish. 1998. Mitochondrial cytochrome oxidase I gene sequences support an Asian origin for the Portuguese oyster *Crassostrea angulata*. *Marine Biology* **131**: 497-503.

Ó Foighil, D., T. J. Hilbish, and R. M. Showman. 1996. Mitochondrial gene variation in *Mercenaria* clam sibling species reveals a relict secondary contact zone in the western Gulf of Mexico. *Marine Biology* **126**: 675-683.

Palumbi, S. R., A. Martin, S. Romano, W. O. McMillan, L. Stice, and G. Grabowski. 1991. *The Simple Fool's Guide to PCR*. University of Hawaii, Honolulu [Privately distributed].

Pfenninger, M. and F. Magnin. 2001. Phenotypic evolution and hidden variation in *Candidula unifasciata* ssp. (Helicellinae, Gastropoda) inferred by 16S variation and quantitative shell traits. *Molecular Ecology* **10**: 2541-2554.

Rawson, P. D. and T. J. Hilbish. 1995. Evolutionary relationships among the male and female mitochondrial DNA lineages in the *Mytilus edulis* species complex. *Molecular Biology and Evolution* **12**: 893-901.

Renard, E., V. Bachmann, L. Cariou, and C. Moreteau. 2000. Morphological and molecular differentiation of invasive freshwater species of the genus *Corbicula* (Bivalvia, Corbiculidea) suggest the presence of three taxa in French rivers. *Molecular Ecology* **9**: 2009-2016.

Rosenberg, N. A. 2003. The shapes of neutral gene genealogies in two species: Probabilities of monophyly, paraphyly, and polyphyly in a coalescent model. *Evolution* **57**: 1465-1477.

Ross, T. K. 1999. Phylogeography and conservation genetics of the Iowa Pleistocene snail. *Molecular Ecology* **8**: 1363-1373.

Simison, W. B. and D. R. Lindberg. 1999. Morphological and molecular resolution of a putative cryptic species complex: A case study of *Notoacmea fascicularis* (Menke, 1851) (Gastropoda: Patellogastropoda). *Journal of Molluscan Studies* **65**: 99-109.

Stepien, C. A., A. N. Hubers, and J. L. Skidmore. 1999. Diagnostic genetic markers and evolutionary relationships among invasive dreissenoid and corbiculoid bivalves in North America: Phylogenetic signal from mitochondrial 16s rDNA. *Molecular Phylogenetics and Evolution* **13**: 31-49.

Swofford, D. L. 2002. *PAUP*: Phylogenetic Analysis Using Parsimony (And Other Methods) 4.0 Beta*. Sinauer Associates, Sunderland, Massachusetts [CD-ROM].

Takahata, N. and M. Nei. 1985. Gene genealogy and variance of interpopulational nucleotide differences. *Genetics* **110**: 325-334.

Tatusova, T. A. and T. L. Madden. 1999. Blast 2 sequences-a new tool for comparing protein and nucleotide sequences. *FEMS Microbiology Letters* **174**: 247-250.

Thacker, R. W. and M. G. Hadfield. 2000. Mitochondrial phylogeny of extant Hawaiian tree snails (Achatinellinae). *Molecular Phylogenetics and Evolution* **16**: 263-270.

Thomaz, D., A. Guiller, and B. Clarke. 1996. Extreme divergence of mitochondrial DNA within species of pulmonate land snails. *Proceedings of the Royal Society of London B* **263**: 363-368.

Wilding, C. S., J. Grahame, and P. J. Mill. 2000. Mitochondrial DNA CoI haplotype variation in sibling species of rough periwinkles. *Heredity* **85**: 62-74.

Wilke, T. and G. M. Davis. 2000. Infraspecific mitochondrial sequence diversity in *Hydrobia ulvae* and *Hydrobia ventrosa* (Hydrobiidae: Rissooidea: Gastropoda): Do their different life histories affect biogeographic patterns and gene flow? *Biological Journal of the Linnean Society* **70**: 89-105.

Wilke, T., E. Rolán, and G. M. Davis. 2000. The mudsnail genus *Hydrobia* s.s. in the northern Atlantic and western Mediterranean: A phylogenetic hypothesis. *Marine Biology* **137**: 827-833.

Accepted: 24 February 2004